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Evaluation of Capsular Polysaccharide Production by *Haemophilus Influenzae* type B in Different Culture Media

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ABSTRACT

Haemophilus influenzae b (Hib), an encapsulated Gram-negative coccobacillus, is one of the most common agents of meningitis worldwide. The Hib vaccine is included in the routine immunization schedule of several countries. The capsular polysaccharide, Poliribosyl-Ribitol-Phosphate (PRP), conjugated to a carrier protein is the antigen of the vaccine against Hib. Currently in industrial processes, Hib is cultivated in a soy-peptone and yeast-extract based complex medium (MMP). This work aimed to evaluate whether a chemically defined medium could replace the MMP medium in the PRP production. The use of a defined media could offer several advantages in the whole process and would be of great interest to the industrial PRP production in vaccination programs. The PRP production ranged from 100 to 290 mg L^{-1} in the studied semi-defined and defined media. The formulation of several chemically defined media based on literature led to a promising composition as is the case of medium G which presented a production of 295 mg $PRPL^{-1}$ with a lower biomass value of 4.8 UA ($O.D._{540\ nm}$) when compared to MMP medium with 395 mg $PRPL^{-1}$ and 6.45 UA respectively.

INTRODUCTION

Haemophilus influenzae type b (Hib) is a Gram-negative, rod-shaped, pleomorphic, and facultative anaerobic bacterium that inhabits the human respiratory tract. Of the six known *H. influenza* (Hi) serotypes (a–f) [1], type b is known to be the most virulent strain, causing pneumonia, bacteremia, and meningitis, generating neurological sequelae in affected surviving children, elderly and immunosuppressed [2,3].The capsular polysaccharide, Poliribosyl-Ribitol-Phosphate (PRP), is the main virulence factor from Hib strain and it is also a powerful antigen for Hib vaccine [4]. The second-generation Hib vaccine, which induces a T-cell dependent immune response, was produced as a PRP-protein conjugate and licensed in the United States in 1987 [5,6]. Before the introduction of a Hib vaccine in Brazil, held in 1999, the Hib spread was the most common cause of meningitis in children less than two years old and had a large impact on child mortality [7]. Nowadays, the Hib vaccine is included in the routine immunization schedule of more than 100 countries [3]. International health agencies, such as the World Health Organization (WHO), have been encouraging vaccination against Hib in developing countries through polyvalent vaccines, such as the combination with tripledipthteria, tetanus and pertussis (DTP) vaccine, hepatitis B vaccine (HepB), and polio vaccine through the Hib Initiative Program, along with the Global Alliance for Vaccine Innovation (GAVI) [8,9].

H. influenza is considered a fastidious microorganism and it has a deficiency in its enzymatic system, requiring micronutrients and some specific components for its growth, also known as factors V and X [10]. Factor V is nicotinamide adenine dinucleotide coenzyme (NAD), which is essential for oxidation-reduction processes in cell metabolism and it is necessary due to the absence of NAD biosynthetic pathways [11,12]. Factor X is hemin, a protoporphyrin IX containing a ferric ion, required for the synthesis of cytochrome c and other iron-containing respiratory enzymes [12].

As a result of its specific micronutrients requirement, complex culture media have been widely used in the literature to promote Hib growth. The culture medium described in the literature for industrial-scale production of the capsular polysaccharide of *H. influenzae* type b (MMP) consists of soy peptone, a vegetal origin compound, as the main nitrogen source and yeast extract supplemented with glucose, hemin, NAD, and inorganic salts [13,14]. Other media containing compounds of animal origin have been reported, such as casein hydrolysate [15,16]. Even though MMP medium provides high PRP production, complex media containing animal origin components are unsuitable for vaccine production due to the risk of contamination with viruses, mycoplasma, and prions [17].

The use of chemically defined media offers several advantages when compared to complex media, such as process reproducibility improvement once all of the chemical components would be known and have a constant concentration, which would allow a consistent performance, avoiding batch-to-batch variation. A defined medium can reduce the cost of large-scale processes, besides enabling studies of the metabolic pathways, nutritional needs for growth, and synthesis of the product of interest. Furthermore, it also offers an advantage for scaling up the purification process and in the analysis of the final products [18].

Studies on Hib cultivation processes using a chemically defined media are rather scarce in the literature. Different compositions of defined media are reported for *H. influenzae* to study of genetics and developmental competence of the microorganism [19-23].In this context, the objective of this work is to establish the composition of a chemically defined medium for cellular growth and production of *Haemophilus influenzae* type b capsular polysaccharide.

Table 1: Evaluation of different media for the development of *H influenzae* type b and synthesis of capsular polysaccharide.

	Medium							
Component	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
NaCl	5	5	5	5	5	5	5	5.8
K_2HPO_4	2.5	2.5	2.5	2.5	2.5	3.5	3.5	3.5
Na_2HPO_4	13.1	13.1	13.1	13.1	13.1	-	-	-
$NaH_2PO_4*H_2O$	3.3	3.3	3.3	3.3	3.3	-	-	-
Glucose	5	5	5	5	5	-	-	-
Yeast Extract	5	5	-	-	-	-	-	-
Bacto Soytone	10	-	-	-	-	-	-	-
NAD	0.015	0.015	0.015	0.015	0.015	0.004	0.007	0.004
Haemin	0.03	0.03	0.03	0.03	0.03	0.03	0.015	0.03
L-Cystine	-	0.04	0.05	0.05	0.05	0.05	0.3	0.2
L-Aspartic acid	-	0.55	0.82	0.82	0.82	0.82	0.81	0.5
L-Glutamic acid	-	0.89	1.39	1.39	1.39	1.39	0.51	1.3
L-Arginine	-	0.28	0.41	0.41	0.41	0.41	0.2	0.3
Glycine	-	0.21	0.36	0.36	0.36	0.36	0.35	0.03
L-Lysine	-	0.29	0.52	0.52	0.52	0.052	0.45	0.05
L-Methionine	-	0.05	0.09	0.09	0.09	0.09	0.2	0.1
L-Serine	-	0.14	0.23	0.23	0.23	0.23	0.3	0.1
L-Histidine	-	0.11	0.17	0.17	0.17	0.17	0.2	0.01
L-Leucine	-	0.43	0.67	0.67	0.67	0.67	0.5	0.3
L-Tyrosine	-	0.13	0.17	0.17	0.17	0.17	0.065	0.2
L-Glutamine	-	0.01	0.03	0.03	0.03	0.03	0.75	0.3
L-Isoleucine	-	0.28	0.47	0.47	0.47	0.47	0.4	0.05
L-Tryptophan	-	0.02	0.05	0.05	0.05	0.05	0.07	0.005
L-Valine	-	0.27	0.48	0.48	0.48	0.48	0.3	0.02
L-Threonine	-	0.11	0.19	0.19	0.19	-	-	-
L - Phenylalanine	-	0.31	0.49	0.49	0.49	0.49	0.625	0.015
L-Proline	-	0.2	0.3	0.3	0.3	-	-	-
L-Cysteine	-	-	-	-	-	0.12	0.15	0.12
Uracil	-	-	0.1	0.1	0.1	0.1	0.2	0.1
Hypoxanthine	-	-	0.02	0.02	0.02	0.02	0.015	0.02
Inosine	-	-	2	4	2	2	1.125	2
Thiamine	-	-	0.004	0.008	0.004	0.004	0.01	0.0045
Pantothenic acid	-	-	0.0037	0.0074	0.0037	0.0037	0.003	0.004
NH_4Cl	-	-	0.022	0.022		0.22	0.22	0.22
$MgSO_4*7H_2O$	-	-	0.5	0.5	0.5	0.43	0.43	0.43
$CaCl_2*2H_2O$	-	-	0.029	0.029	0.029	-	-	-
EDTA	-	-	0.004	0.004	0.004	0.004	0.004	0.004
Tween 80	-	-	0.02	0.02	0.02	0.02	0.02	0.02
Sodium lactate	-	-	1.6	1.6	1.6	1.6	1.6	1.6
Glycerol	-	-	-	-	-	5	5	5
PVA	-	-	-	-	-	0.02	0.02	0.02
$CaCl_2$	-	-	-	-	-	0.029	0.165	0.029
K_2SO_4	-	-	-	-	-	1	1	1
KH_2PO4	-	-	-	-	-	2.7	2.7	2.7

Components and concentrations were set according to related media in literature and components com complex medium. NAD (Nicotinamide adenine dinucleotide), PVA (Polyvinyl alcohol).

MATERIALS AND METHODS

Strain

Haemophilus influenzae type b strain GB3291 was acquired from the Núcleo de Coleção de Micro-organismos of the Instituto Adolph Lutz, São Paulo. The working seed was prepared according to Takagi et al, 2006 and stored in liquid nitrogen.

Media composition

Complex medium, semi-defined and defined media were used in this study according to Table 1: A) complex medium MMP [13] as control; B) Bacto Soytone (BD) was replaced for its related constituent amino acids; C) both Bacto Soytone (BD) and Yeast Extract (BD) were replaced by its respective constituent amino acids and, vitamins and inosine were added according to concentrations described by Herriot et al.; D) modified medium C with vitamin concentrations and inosine doubled; E) modified medium E without NH_4Cl.

After a literature review [19,22,23], 36 components were selected and their concentrations were set using 2 criteria: F) only the concentrations of amino acids were equal to medium C; G and H) all component concentrations were set according to literature [22].

Shake Flasks Experiments

Pre-inoculum was prepared by transferring 400 µL of bacterial suspension (1.9 x 10^9 CFU mL^{-1}) into a 300 ml Erlenmeyer flask containing 50 ml of culture medium and incubated under static anaerobic condition at 37°C for 6 hours. A volume of the bacterial culture resulting in an optical density of 0.05 AU at 540 nm ($OD_{540\ nm}$) was transferred to a 500 mL Erlenmeyer flask containing 100 mL of medium was incubated on a rotative shaker (250 RPM) at 37 °C for 12 hours. A volume of bacterial culture corresponding to the initial OD of 0.1 AU was used as inoculum and the experiments were performed for 12 hours on a rotatory shaker (300 RPM) at 37°C in a 500 mL Erlenmeyer flask containing 100 mL of sterile culture medium.

Analysis

Cell growth: To monitor cell growth, samples were collected each one hour during the cultivation to measure the optical density at 540 nm. Samples were diluted with 0.85% NaCl when OD_{540nm} exceeded 0.5 AU, ensuring uniform distribution of the cells insuspension following Lambert-Beer's law.

Capsular polysaccharide determination: PRP concentration was measured by high anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [24]. The samples were diluted in deionized water to 540 µL and 180 µL of NaOH 400 mmol L^{-1}. The mixture was incubated at 37 °C for 20 h under alkaline hydrolysis of polysaccharide and neutralized with 180 µL of 400 mmol L^{-1} acetic acid. A 100 µL of glucose-6-phosphate at 100 µmol L^{-1} was used as an internal standard. A volume of 10 µL of this mixture was injected into the anion exchange column CarboPac PA-10 coupled to pre-column AminoTrap, mounted on the ICS5000 chromatographic system (Thermo Fisher Scientific Inc.). The methodology included the gradients of NaOH and sodium acetate described in by Haan et al., in addition to the electrochemical potentials defined by the authors for the gold electrode. The calibration curve was plotted using a purified PRP standard at 30 mg L^{-1} in the range of 1 to 12 mg L^{-1}.

RESULTS AND DISCUSSION

H. influenzae type b is a fastidious bacteria and due to its metabolic needs, the culture medium described in the literature for industrial-scale production of capsular polysaccharide consists of soy peptone and yeast extract, besides glucose, hemin, NAD, and inorganic salts (MMP medium). Figure 1 shows the Hib growth (a) and PRP production (b) in a time course of 12hours considering ten independent shake flask experiments. As expected, experiments presented a high variation of both studied parameters and low reproducibility.

Experiments were performed to evaluate whether components of complex media could be replaced by simple components such as amino acids and vitamins. The elaboration of the defined medium was carried out in two ways:

1) replacing only Bacto Soytone (B) and both Bacto Soytone and Yeast extract at the same time (C - E) for its related constituent amino acids and supplemented with vitamins, salts lactate as reported by Herriot [22]. Considering that medium D

has the double concentration of inosine, thiamin, and pantothenic acid than C, and medium E had the same composition as medium C, but without NH_4Cl; 2) media F-Hwere formulated based on the media reported previously for several strains of *Haemophilus influenza* [19,22,23] and amino acid concentrations in MMP medium [13]. For this purpose, 36 components and their concentrations were selected from the literature. Amino acids concentrations of medium F were equal to medium C. Figure 2 shows the results of cultivation with media A - H. The media in which Bacto Soytone was replaced (B) did not result in cell growth; this indicates thatits components play an essential role in cell development. In the media where both Bacto Soytone and Yeast Extract were replaced by the respective amino acids (C,D, and E) and supplemented with salts and vitamins as reported in literature [22], the measured OD_{540nm} ranged from 4.0 to 2.0. Although the supplementation with ammonium chloride seems to increase nitrogen uptake [22], its absence in medium E does not compromise the growth. On the other hand, doubling inosine, thiamin and pantothenic acid slightly decreased cell growth and PRP synthesis. Analyzing polysaccharide production (Figure 2b), the medium MMP (A) showed the highest PRP production of 350 mg L^{-1}, and defined medium G reach a production of 290 mg L^{-1}.

Media C, D, E reached a PRP production close to 150 mg L^{-1}. In these media, inosine played a fundamental role since in its absence affects the growth, reflecting also in the polysaccharide production as in medium B. According to literature [25], inosine is the only nucleoside added to the medium (except for NAD), which acts as a growth co-factor. After being degraded into hypoxanthine, it favors the incorporation of thymine and thymidine by *Haemophilus influenza* [25]. The obtained data indicate that the same could happen with the uracil nucleotide present in the tested medium, resulting in less growth in the absence of inosine. Therefore, the decrease in the growth rate in the absence of this component signals that *Haemophilus influenzae* does not have the genetic machinery for making nucleotides without the presence of at least one ribonucleotide. Inosine has also been described as an essential component for the development of *Haemophilus influenzae* competence [21,25]. In general, defined media F-H had a better performance than defined and semi-defined media B-E based on complex medium A (Figure 2). A major difference between these media compositions is the main carbon source which is glucose for media A-E and glycerol and sodium lactate in media F-H. These results could indicate that glycerol is a better carbon source than glucose in defined media. It has been shown that glucose uptake in *H. influenzae* depends on an ATP-dependent kinase, in which glucose is phosphorylated and fed into the central metabolism [26]. Then, glucose is fully oxidized into pyruvate through the glycolytic pathway [26]. Acetate is the main product during aerobic growth and formate is the major product during anaerobic growth [27]. Moreover, acetate was found as the major by-product accumulated in the broth during batch fermentations of *Haemophilus influenzae* type b, decreasing the medium pH and inhibiting cellular growth [13].

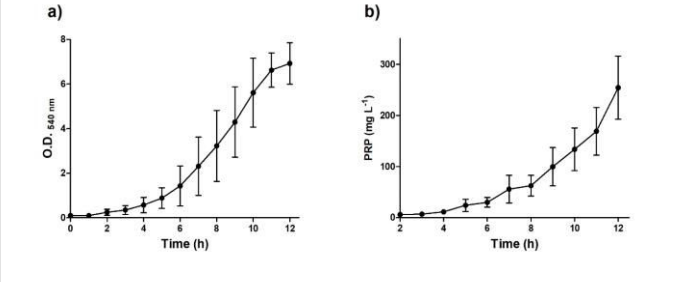

Figure 1: *Haemophilus influenzae* type b (Hib) growth and PRP production in shake flasks cultivations carried out with complex media. a) Hib growth is given by Optical Density (O.D.) at 540 nm; b) Poliribosyl-ribitol-phosphate (PRP) production. Graphs represent the mean and Standard Deviation (SD) of ten independent experiments.

The glycerol uptake in *H.influenzae* depends on the expression of the *glp F* gene that facilitates the diffusion of glycerol thought the membrane and the expression of a glycerol-3-phosphate transporter [26]. Glycerol is converted into dihydroxyacetone phosphate (DHAP) and it isfed into the glycolytic pathway [26]. Studies have shown that L-lactate had a stimulatory effect on the development of competence [28] and cellular growth [29] of Hi strains, which indicates that lactates could be a carbon-source. Beyond that, not much more is known about lactate metabolism in *H. influenza* [30].

Media F-H have been supplemented with L-cysteine which appears to be an essential sulfur source for *H. influenzae* strains [31]. Arginine, glutamate, pantothenate, thiamin, uracil, and inorganic phosphate have also been determined as minimal substrate requirements for bacterial growth in Rd strains [31].

In a recent study, Farshad and Pour have studied the PRP production in different complex media by Hib. The authors' results have indicated that PRP production is greatly dependent on not only the kind of growth medium but also on various medium components [32]. The differences between Hib growth and PRP production obtained by media F-H (Figure 2c,d) supportthe possibility of achieving a PRP production similar to the obtained by using complex medium through altering the concentration of essential medium components, as shown in Table 2. Medium Gindicates a promissory result once it was possible to achieve a PRP production close to the one obtained in complex medium but with a smaller cellular growth, which could facilitate the purification process.

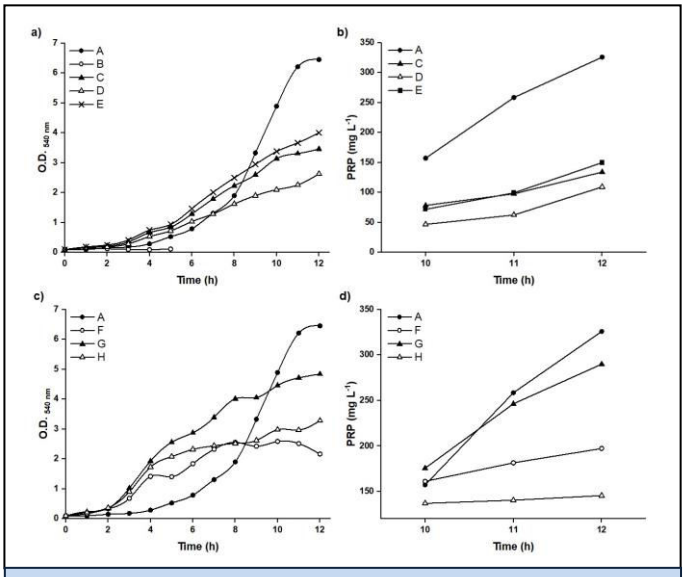

Figure 2: *Haemophilus influenzae* type b (Hib) growth and PRP production in shake flasks cultivations carried out with semi-defined and defined media. a and c) Hib growth is given by Optical Density (O.D.) at 540 nm; b and d) Poliribosyl-ribitol-phosphate (PRP) production in mg L^{-1}. Graphs a and c are related to media: A, B, C, D, and E, and graphs b and d are related to media A, F, G, and H.

Table 2: Comparison of OD_{540nm} and PRP synthesis among complex medium MMP (A) and three promising different compositions of defined media.

Medium	$O.D._{540nm}$	PRP (mg L^{-1})
A	6.45	350
F	2.1	200
G	4.8	290
H	3.2	145

CONCLUSION

Complex medium MMP is used traditionally in the production of capsular polysaccharide PRP for vaccine production. However, working with complex compound result in high deviation in the cell growth and PRP synthesis among different lots. In summary, this work sought to evaluate whether a chemically defined media in *Haemophilus influenzae* type b cultivations could replace complex media MMP, aiming to produce Poliribosyl-Ribitol-Phosphate (PRP), the antigen for Hib vaccine. It is known that chemically defined media can offer several advantages in industrial processes, mainly for vaccine production. By replacing yeast extract and soy-peptone for its related amino acids, we have observed a decrease in Hib growth and PRP production. 36 components were selected from defined media reported in the literature, it was possible to achieve promising results as the medium G which resulted in 295 mg $PRPL^{-1}$, very close to the production of complex medium of 395 mg $PRPL^{-1}$. Moreover, by altering the concentration of these 36 components in these media, differences in cellular growth and PRP production were observed. Additional studies to investigate the effect of each of these selected components seeking to establish an optimal composition are in progress. As a complex system such as bacteria metabolism, studying and optimizing components of a defined medium will require statistical tools to evaluate the obtained results.

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